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Specific α-galactosidase inhibitors, *N*-methylcalystegines

Structure/activity relationships of calystegines from *Lycium chinense*

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An examination of the roots of *Lycium chinense* (Solanaceae) has resulted in the discovery of 14 calystegines, a cycloheptane bearing an amino group and three hydroxyl groups, and two polyhydroxylated piperidine alkaloids. Calystegines A_7 and B_5, in addition to the previously known calystegines A_3, A_5, A_6, B_1, B_2, B_3, B_4, C_1, C_2 and N_1, were isolated and determined as 1α,2β,4α-trihydroxy-nortropane and 1α,2α,4α,7α-tetrahydroxy-nortropane, respectively. *L. chinense* also had two polyhydroxytropanes bearing a methyl group on the nitrogen atom, unlike the previously reported nortropane alkaloids. They were established as *N*-methylcalystegines B_2 and C_1, and their *N*-methyl groups were found to be axially oriented from NOE experiments. 1β-Amino-3β,4β,5α-trihydroxycycloheptane was also present in *L. chinense* and may be a biosynthetic precursor of the calystegines that occur in this plant. Two polyhydroxypiperidine alkaloids, fagomine and 6-deoxyfagomine, were isolated. Calystegine B_2 is a potent competitive inhibitor of almond β-glucosidase (K_i = 1.9 μM) and coffee bean α-galactosidase (K_i = 0.86 μM), while *N*-methylcalystegine B_2 was a more potent competitive inhibitor of the latter enzyme (K_i = 0.47 μM) than the parent compound but showed a marked lack of inhibitory activities towards most other glycosidases. Since this compound is a very specific inhibitor of α-galactosidase and inhibits rat liver lysosomal α-galactosidase with a K_i of 1.8 μM, it may provide a useful experimental model for the lysosomal storage disorder, Fabry's disease. The addition of a hydroxyl group at C6exo, as in calystegines B_1 and C_1, enhances the inhibitory potential towards β-glucosidase and β-galactosidase but markedly lowers or abolishes inhibition towards α-galactosidase. Hence, the N-methylation of calystegine C_1 did not enhance its inhibition of α-galactosidase. The chemical N-methylation of calystegines A_3 and B_4 markedly enhanced inhibition of coffee bean α-galactosidase, with K_i values of 5.2 μM and 36 μM, respectively, but almost eliminated their inhibitory potential towards β-glucosidase and trehalase, respectively. Thus, methylation of the nitrogen atom significantly altered the specificity of the inhibitors.

Keywords: *Lycium chinense*; α-galactosidase inhibitor; *N*-methylcalystegine; chemical N-methylation; alteration of specificity.

In 1988, Tepfer et al. (1988) reported the presence of a group of compounds in underground organs and root exudates of *Calystegia sepium* (Convolvulaceae). Systematic screening of a number of plant species, representing 26 families, revealed their presence in *Convolvulus arvensis* (Convolvulaceae) and *Atropa belladonna* (Solanaceae) as well (Tepfer et al., 1988). These compounds were named calystegines and proposed as nutritional mediators for rhizosphere bacteria, establishing and maintaining specific plant-bacterium relationships. Calystegines were characterized as nortropane alkaloids with a high degree of hydroxylation and an unusual aminoketal functionality at the bridgehead position (Ducrot and Lallemand, 1990; Goldmann et al., 1990). Calystegines have been classified into three groups A, B and C on the basis of the number of hydroxyl group substituents on the nortropane ring, i.e. tri-, tetra- and penta-hydroxy-nortropane alkaloids, respectively. Recently, we reported a class of calystegine with a bridgehead amino group in the place of a bridgehead hydroxyl group from *Hyocyamus niger*, which we designated calystegine N_1 (Asano et al., 1996a). Calystegine N_1 is non-enzymically converted into calystegine B_2 with a bridgehead hydroxyl group on storage.

Calystegines have been shown to occur in the plant families Convolvulaceae, Solanaceae and Moraceae (Molyneux et al., 1996). However, the greatest number of plant species producing calystegines belong to the family Solanaceae. Tropane alkaloids, bearing a methyl substituent on the nitrogen atom, are a well-recognized group of structurally related natural products and include such important medicinal alkaloids as cocaine, scopolamine and atropine (Lounasmaa, 1988; Lounasmaa and Tamminen, 1993). Tropane alkaloids have long been known to have anticholinergic, antiemetic, parasympatholytic, anesthetic and many other pharmacological actions. The genera *Atropa*, *Da-*

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Abbreviations. HRFAB, high-resolution fast-atom bombardment; IL, interleukin.

Enzymes. α-Glucosidase (EC 3.2.1.20); β-glucosidase (EC 3.2.1.21); α-galactosidase (EC 3.2.1.22); β-galactosidase (EC 3.2.1.23); α-mannosidase (EC 3.2.1.24); β-mannosidase (EC 3.2.1.25); α,α-trehalase (EC 3.2.1.28); α-L-fucosidase (EC 3.2.1.51).

tura, *Duboisia*, *Hyoscyamus* and *Scopolia* of the Solanaceae are especially rich sources of hyoscyamine and/or scopolamine. Calystegines have been found in all of these genera, and *Atropa belladonna* contained higher amounts of calystegines A_3, B_1 and B_2 in aerial parts than in roots (Dräger et al., 1995). Low amounts of calystegines were detected in *Datura wrightii* leaves (Nash et al., 1993) and *Datura stramonium* roots and leaves (Dräger et al., 1995). We have recently isolated calystegines A_3, A_5, A_6, B_1, B_2, B_3 and N_1 from *H. niger* (Asano et al., 1996a), calystegines A_3, A_5, B_1, B_2, B_3, B_4 and C_1 from *Scopolia japonica* roots (Asano et al., 1996b) and calystegines B_1, B_2, B_4, C_1 and C_2 from *Duboisia leichhardtii* leaves (Kato et al., 1997a).

Calystegines are known to be present in edible vegetables such as potatoes (*Solanum tuberosum*), egg-plant (*Solanum melongena*) and sweet potatoes (*Ipomoea batatas*; Nash et al., 1993, 1996; Dräger, 1995; Asano et al., 1997). Dräger et al. (1995) have reported that the calystegine concentration can vary greatly within one plant and that the calystegine B_2 content is 400 μg/g tissue in sprouts growing from greening potato tubers that were kept in daylight for two weeks. It is known that calystegine B_2 is a potent competitive inhibitor of β-glucosidases and α-galactosidases with K_i values of 10^{-6}–10^{-7} M, and calystegines B_1 and C_1 are potent competitive inhibitors of almond β-glucosidase, with K_i values of 1.8 μM and 0.45 μM, respectively (Molyneux et al., 1993; Asano et al., 1994a,b, 1995). It is therefore not unreasonable to predict that the calystegines, which inhibit β-glucosidase and α-galactosidase, would produce syndromes that mimic the genetic absence or diminution of such activities, namely Gaucher's and Fabry's diseases, respectively (Molyneux et al., 1994, 1996). Furthermore, calystegines were contained in an oriental crude drug, Radix Physalis, made from the roots of *Physalis alkekengi* var. *francheti* (Solanaceae), which has been used as an antitussive and diuretic drug (Asano et al., 1995). Calystegines A_3, A_5, B_1, B_2 and B_3 were isolated from this crude drug. Our search for calystegines in crude drugs of the plant family Solanaceae led to the discovery of 14 calystegines and several related alkaloids from Lycii Radicis Cortex, the root barks of *Lycium chinense*. Among these calystegines, were the *N*-methylcalystegines B_2 and C_1. In this paper we report the isolation of 14 calystegines and several related alkaloids, the structural determination of calystegines and related alkaloids, and the structure/activity relationships of calystegines. In addition, we report the preparation of unnatural *N*-methylcalystegines A_3, B_3 and B_4 and their glycosidase-inhibitory activities.

EXPERIMENTAL PROCEDURES

General methods. The purity of samples was checked by high-performance TLC Silica Gel-60F_{254} (Merck) using the solvent system PrOH/AcOH/H_2O (4:1:1) and were detected by spraying with chlorine/*o*-tolidine reagent. The purity of the compounds was also established by GC/MS of the alkaloids as their trimethylsilyl derivatives. Optical rotations were measured with a Jasco DIP-370 digital polarimeter. ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) spectra were recorded on a Jeol JNM-GX 400 spectrometer, and ^{13}C-NMR (22.5 MHz) spectra on a Jeol JNM-EX 90 A spectrometer as indicated, in ^2H_2O with sodium 3-(trimethylsilyl)-(2,2,3,3-$^2H_4$)propionate, or in $C^2H_3O^2H$ with tetramethylsilane as the internal standard. Chemical shifts are reported as δ values in ppm, and the coupling constants (*J*) are given in Hz. Mass spectra were measured on a Jeol JMS-SX 102A spectrometer.

Materials. The enzymes α-glucosidase (from rice), β-glucosidase (from almonds), α-galactosidase (from green coffee beans), β-galactosidase (from bovine liver), α-mannosidase (from jack beans), β-mannosidase (from snail), trehalase (from porcine kidney) and α-L-fucosidase (from bovine epididymis), and the *p*-nitrophenyl glycoside and disaccharide substrates were purchased from Sigma Chemical Co. The partially purified lysosomal fraction prepared by the procedures of Tsuji et al. (1977) was used as a source of rat liver lysosomal glycosidases. Enzyme activities were assayed by the methods described in our previous paper (Asano et al., 1995).

Kinetics of inhibition. Kinetic parameters were determined by the double-reciprocal-plot method of Lineweaver-Burk at increasing concentrations of the appropriate *p*-nitrophenyl glycoside. The reaction was stopped by the addition of 0.4 M Na_2CO_3. The amount of *p*-nitrophenol released was measured by the absorbance at 400 nm. The activity of pig kidney trehalase was measured with trehalose as substrate and the reaction was stopped by boiling for 3 min. Released glucose was measured by a glucose oxidase/peroxidase kit (Glucose B-test; Wako).

Isolation of polyhydroxylated alkaloids. The roots (5 kg) of *L. chinense* from a commercial source were extracted three times with hot water for 2 h. The hot-water extracts (38 l) were applied to a column of Amberlite IR-120B (H^+, 1 l) and eluted with 0.5 M NH_4OH. The eluate was concentrated to give a brown oil (10 g). This oil was applied to a Dowex 1-X2 column (OH^-, 200 ml) and eluted with 1 l water. The concentrated eluate was chromatographed over an Amberlite CG-50 column (2.4 cm×90 cm, NH_4^+) with water (fraction size, 19 ml). The water eluate was divided into five pools, A (fractions 15–27, 1.85 g protein), B (fractions 28–34, 197 mg), C (fractions 35–52, 84 mg), D (fractions 53–65, 50 mg), and E (fractions 90–106, 51 mg). The 0.5 M NH_4OH eluate from the same column was designated pool F (122 mg). Pool A was further chromatographed on a Dowex 1-X2 column (1.9 cm×92 cm, OH^-) with water as an eluant to give calystegine N_1 (27 mg), *N*-methylcalystegine B_2 (9 mg), calystegines B_2 (630 mg), B_1 (64 mg), and C_2 (8 mg), *N*-methylcalystegine C_1 (15 mg), and calystegine C_1 (184 mg), in order of elution. Other pools were chromatographed on a Dowex 1-X2 column (1.9 cm×92 cm, OH^-) with water as an eluant to give calystegine B_3 (87 mg) from pool B, calystegines B_4 (4 mg), A_7 (8 mg), A_5 (5 mg) and A_6 (6 mg) in order of elution from pool C, calystegine A_3 (32 mg) from pool D, fagomine (34 mg) from pool E, and 6-deoxyfagomine (7 mg), calystegine B_5 (11 mg) and 1β-amino-3β,4β,5α-trihydroxycycloheptane (9 mg) in order of elution from pool F.

Calystegine A_7 (1α,2β,4α-trihydroxynortropane). $[\alpha]_D$ −10.8° (*c* 0.27, H_2O); high-resolution fast-atom-bombardment (HRFAB) MS *m/z* 160.0971 $[M + H]^+$ ($C_7H_{14}O_3N$ requires 160.0974); ^{1}H-NMR (400 MHz, ^2H_2O) δ = 1.44 (m, 1H, H6endo), 1.53 (m, 1H, H7exo), 1.61 (ddd, 1H, $^3J_{2,3ax}$ 9.5, $^3J_{3ax,4}$ 4.0, $^2J_{3ax,3eq}$ 14.7 Hz, H3ax), 1.99 (dddd, 1H, $^3J_{2,3eq}$ 5.5, $^3J_{3eq,4}$ 2.2, $^2J_{3ax,3eq}$ 14.7, $^4J_{3eq,5}$ 1.8 Hz, H3eq), 2.05 (m, 1H, H7endo), 2.10 (m, 1H, H6exo), 3.33 (m, 1H, H5), 3.80 (m, 1H, H4) 3.84 ppm (ddd, 1H, $^3J_{2,3ax}$ 9.5, $^3J_{2,3eq}$ 5.5, $^4J_{2,7exo}$ 1.8 Hz, H2); ^{13}C-NMR (22.5 MHz, ^2H_2O) δ = 26.2 (C6), 29.1 (C7), 36.6 (C3), 60.9 (C5), 73.0 (C4), 73.7 (C2) and 93.7 ppm (C1).

Calystegine B_5 (1α,2α,4α,7α-tetrahydroxynortropane). $[\alpha]_D$ +9.6° (*c* 0.31, H_2O); HRFAB MS *m/z* 176.0923 $[M + H]^+$ ($C_7H_{14}O_4N$ requires 176.0923); ^{1}H-NMR (400 MHz, ^2H_2O) δ = 1.77 (ddt, 1H, $^3J_{2,3eq} = {}^3J_{3eq,4}$ = 2.1, $^2J_{3ax,3eq}$ 16.3, $^4J_{3eq,5}$ 1.8 Hz, H3eq), 1.84 (ddd, 1H, $^3J_{5,6exo}$ 7.6, $^3J_{6exo,7}$ 3.4, $^2J_{6endo,6exo}$ 14.5 Hz, H6exo), 1.89 (dt, 1H, $^3J_{2,3ax} = {}^3J_{3ax,4}$ = 4.3, $^2J_{3ax,3eq}$ 16.3 Hz, H3ax), 2.11 (dd, 1H, $^2J_{6endo,6exo}$ 14.5, $^3J_{6endo,7}$ 7.7 Hz, H6endo), 3.38 (ddd, 1H, $^4J_{3eq,5}$ 1.8, $^3J_{4,5}$ 3.3, $^3J_{5,6exo}$ 7.6 Hz, H5), 3.58 (m, 1H, H4), 3.71 (dd, 1H, $^3J_{2,3eq}$ 2.1, $^3J_{2,3ax}$ 4.3 Hz, H2) and 3.83 ppm (dd, 1H, $^3J_{6endo,7}$ 7.7, $^3J_{6exo,7}$ 2.4 Hz, H7); ^{13}C-NMR

(100 MHz, ^2H_2O) δ = 34.3 (C3), 36.9 (C6), 58.9 (C5), 69.1 (C4), 71.7 (C2, C7) and 92.3 ppm (C1).

***N*-Methylcalystegine B_2 (1α,2β,3α,4β-tetrahydroxytropane).** $[\alpha]_D$ +22.2° (*c* 0.22, H_2O); HRFAB MS *m/z* 190.1078 $[M + H]^+$ ($C_8H_{16}O_4N$ requires 190.1079); ^{1}H-NMR (400 MHz, ^2H_2O) δ = 1.63−1.76 (m, 2H, H6endo, H7exo), 1.96−2.10 (m, 2H, H6exo, H7endo), 2.35 (s, 3H, N-CH_3), 3.19 (dd, 1H, $^3J_{4,5}$ 4.0, $^3J_{5,6exo}$ 7.0 Hz, H5), 3.35 (t, 1H, $^3J_{2,3} = {}^3J_{3,4}$ = 8.5 Hz, H3), 3.48 (dd, 1H, $^3J_{2,3}$ 8.5, $^4J_{2,7exo}$ 1.5 Hz, H2) and 3.72 ppm (dd, 1H, $^3J_{3,4}$ 8.5, $^3J_{4,5}$ 4.0 Hz, H4); ^{13}C-NMR (100 MHz, ^2H_2O) δ = 22.4 (C6), 29.3 (C7), 35.0 (N-CH_3), 65.8 (C5), 74.9 (C4), 78.0 (C3), 78.6 (C2) and 95.9 ppm (C1).

***N*-Methylcalystegine C_1 (1α,2β,3α,4β,6α-pentahydroxytropane).** $[\alpha]_D$ +27.6° (*c* 0.30, H_2O); HRFAB MS *m/z* 206.1028 $[M + H]^+$ ($C_8H_{16}O_5N$ requires 206.1028); ^{1}H-NMR (400 MHz, ^2H_2O) δ = 1.66 (m, 1H, H7exo), 2.49 (s, 3H, N- CH_3), 2.54 (dd, 1H, $^3J_{6,7endo}$ 7.7, $^2J_{7endo,7exo}$ 14.6 Hz, H7endo), 3.14 (br d, 1H, $^3J_{4,5}$ 4.4 Hz, H5), 3.16 (t, 1H, $^3J_{2,3} = {}^3J_{3,4}$ = 8.8 Hz, H3), 3.48 (dd, 1H, $^3J_{2,3}$ 8.8, $^4J_{2,7exo}$ 1.8 Hz, H2), 3.78 (dd, 1H, $^3J_{3,4}$ 8.8, $^3J_{4,5}$ 4.4 Hz, H4), 4.29 ppm (dd, 1H, $^3J_{6,7exo}$ 3.3, $^3J_{6,7endo}$ 7.7 Hz, H6); ^{13}C-NMR (100 MHz, ^2H_2O) δ = 34.8 (N-CH_3), 41.9 (C7), 70.6 (C6), 72.4 (C4), 73.3 (C5), 76.8 (C2), 78.0 (C3) and 95.0 ppm (C1).

1β-Amino-3β,4β,5α-trihydroxycycloheptane. $[\alpha]_D$ +10.1° (*c* 0.30, H_2O); HRFAB MS *m/z* 162.1138 $[M + H]^+$ ($C_7H_{16}O_3N$ requires162.1130); ^{1}H-NMR (400 MHz, $C^2H_3O^2H$) δ = 1.41 (m, 1H, H7β), 1.50 (m, 1H, H6α), 1.77 (m, 1H, H2α), 1.82−1.89 (m, 1H, H6β), 1.89 (ddd, 1H, $^3J_{1,2\beta}$ 8.5, $^3J_{2\beta,3}$ 9.5, $^2J_{2\alpha,2\beta}$ 14.0 Hz, H2β), 1.90−1.96 (m, 1H, H7α), 2.96 (m, 1H, H1), 3.62 (ddd, 1H, $^4J_{2\alpha,4}$ 1.0, $^3J_{3,4}$ 2.7, $^3J_{4,5}$ 5.8 Hz, H4), 3.67 (ddd, 1H, $^3J_{4,5}$ 5.8, $^3J_{5,6\alpha}$ 9.1, $^3J_{5,6\beta}$ 3.0 Hz, H5), 3.93 ppm (dt, 1H, $^3J_{2\alpha,3} = {}^3J_{3,4}$ = 2.7, $^3J_{2\beta,3}$ 9.5 Hz, H3); ^{13}C-NMR (100 MHz, $C^2H_3O^2H$) δ = 30.6 (C6), 33.9 (C7), 40.4 (C2), 51.2 (C1), 71.2 (C3), 75.7 (C5) and 80.8 ppm (C4).

6-Deoxyfagomine. $[\alpha]_D$ −11.1° (*c* 0.1, H_2O); HRFAB MS *m/z* 132.1029 $[M + H]^+$ ($C_6H_{14}O_2N$ requires 132.1025); ^{1}H-NMR (400 MHz, ^2H_2O) δ = 1.17 (d, *J* = 6.4 Hz, 3H, CH_3), 1.48 (ddt, 1H, $^3J_{1eq,2ax}$ 4.6, $^3J_{1ax,2ax} = {}^2J_{2ax,2eq}$ = 12.9, $^3J_{2ax,3}$ 11.5 Hz, H2ax), 1.99 (dddd, 1H, $^3J_{1eq,2eq}$ 2.4, $^3J_{1ax,2eq}$ 2.7, $^2J_{2ax,2eq}$ 12.9, $^3J_{2eq,3}$ 5.1 Hz, H2eq), 2.43 (m, 1H, H5), 2.62 (dt, 1H, $^2J_{1ax,1eq} = {}^3J_{1ax,2ax}$ = 12.9, $^3J_{1ax,2eq}$ 2.7 Hz, H1ax), 2.96 (ddd, 1H, $^2J_{1ax,1eq}$ 12.9, $^3J_{1eq,2ax}$ 4.6, $^3J_{1eq,2eq}$ 2.4 Hz, H1eq), 2.99 (dd, 1H, $^3J_{3,4}$ 9.0, $^3J_{4,5}$ 9.6 Hz, H4), 3.51 (ddd, 1H, $^3J_{2eq,3}$ 5.1, $^3J_{2ax,3}$ 11.5, $^3J_{3,4}$ 9.0 Hz, H3); ^{13}C-NMR (100 MHz, ^2H_2O) δ = 19.9 (C6), 35.5 (C2), 45.4 (C1), 58.2 (C5), 75.7 (C3) and 80.8 ppm (C4).

Preparation of N-methylated derivatives of calystegines. The N-methylated derivatives of calystegines were synthesized by a modification of the method of Kato et al. (1973) as follows. A solution of calystegine in 37% HCHO was mixed with 80% formic acid and stirred at 37°C for 5 days and evaporated. The residue was dissolved in MeOH, applied to an Amberlyst 15 column, washed with MeOH, eluted with 0.5 M NH_4OH, and concentrated. N-methylated derivatives were purified by Dowex 1-X2 (OH^-) chromatography with water as an eluant.

***N*-Methylcalystegine A_3 (1α,2β,3α-trihydroxytropane).** $[\alpha]_D$ −5.6° (*c* 1, H_2O); HRFAB MS *m/z* 174.1129 $[M + H]^+$ ($C_8H_{16}O_3N$ requires 174.1130); ^{13}C-NMR (100 MHz, ^2H_2O) δ = 27.0 (C6), 30.1 (C7), 33.7 (N-CH_3), 37.8 (C4), 60.5 (C5), 72.6 (C3), 79.1 (C2) and 95.4 ppm (C1).

***N*-Methylcalystegine B_3 (1α,2α,3α,4β-tetrahydroxytropane).** $[\alpha]_D$ +51.9° (*c* 0.8, H_2O); HRFAB-MS *m/z* 190.1078 $[M + H]^+$ ($C_8H_{16}O_4N$ requires 190.1079); ^{13}C-NMR (100 MHz, ^2H_2O) δ = 21.2 (C6), 31.1 (C7), 36.2 (N-CH_3), 66.4 (C5), 73.1 (C3), 74.1 (C4), 77.3 (C2) and 99.1 ppm (C1).

***N*-Methylcalystegine B_4 (1α,2β,3α,4α-tetrahydroxytropane).** $[\alpha]_D$ −60.8° (*c* 1.09, H_2O); HRFAB-MS *m/z* 190.1080 $[M + H]^+$ ($C_8H_{16}O_4N$ requires 190.1079); ^{13}C-NMR (100 MHz, ^2H_2O) δ = 24.1 (C6), 27.0 (C7), 36.4 (N-CH_3), 67.4 (C5), 73.9 (C3), 74.0 (C4), 79.1 (C2) and 97.4 ppm (C1).

RESULTS

Structure determination of polyhydroxylated alkaloids. The structures of the polyhydroxylated alkaloids isolated from *L. chinense* were determined by combined ^{1}H-NMR and ^{13}C-NMR spectroscopy, including extensive homonuclear decoupling experiments, NOE enhancements, and two-dimensional ^{1}H-^{13}C COSY or heteronuclear multiple-quantum coherence (Fig. 1). The ^{1}H-NMR and ^{13}C-NMR spectral data of calystegines A_3, A_5, A_6, B_1, B_2, B_3, B_4, C_1, C_2 and N_1 and fagomine isolated from *L. chinense* were completely in accord with those obtained from authentic samples isolated to date (Asano et al., 1994a,b, 1995, 1996a,b; Kato et al., 1997a).

Calystegine A_7 (1α,2β,4α-trihydroxynortropane). The results of ^{13}C-NMR and HRFAB MS (*m/z* 160.0971 $[M + H]^+$, $C_7H_{14}O_3N$ requires 160.0974) analyses indicate that this alkaloid is a trihydroxynortropane. In the ^{1}H-NMR spectrum in ^2H_2O, the coupling patterns of H2 (δ = 3.84 ppm, ddd, $^3J_{2,3ax}$ = 9.5, $^3J_{2,3eq}$ = 5.5, $^4J_{2,7exo}$ = 1.8 Hz) and H3ax (δ = 1.61 ppm, ddd, $^3J_{2,3ax}$ = 9.5, $^3J_{3ax,4}$ = 4.0, $^2J_{3ax,3eq}$ = 14.7 Hz) indicate that H2 and H4 are axial and equatorial, respectively. The ^{1}H-NMR spectral data described above and a W-path long-range coupling between H3eq and H5 ($^4J_{3eq,5}$ = 1.8 Hz) show that the six-membered ring is in a chair conformation. Thus, the relative configuration of calystegine A_7 was determined as 1α,2β,4α-trihydroxynortropane.

Calystegine B_5 (1α,2α,4α,7α-tetrahydroxynortropane). The fifth member of the tetrahydroxylated group, calystegine B_5, had a consistent HRFAB MS value of *m/z* 176.0923 $[M + H]^+$ ($C_7H_{14}O_4N$ requires 176.0923). In the ^{1}H-NMR spectrum in ^2H_2O, the coupling patterns of H2 (δ = 3.71 ppm, dd, $^3J_{2,3ax}$ = 4.3, $^3J_{2,3eq}$ = 2.1 Hz) and H3ax (δ = 1.89 ppm, dt, $^3J_{2,3ax} = {}^3J_{3ax,4}$ = 4.3, $^2J_{3ax,3eq}$ = 16.3 Hz) show that H2 and H4 are equatorial. This was corroborated by NOE effects between H2 and H7 and between H4 and H6endo. Furthermore, NOE effects between H2 and H7 and between H3ax and H7 suggested an endo orientation of H7. Therefore, the relative configuration of calystegine B_5 was shown to be 1α,2α,4α,7α-tetrahydroxynortropane.

N-Methylcalystegine B_2 *(1α,2β,3α,4β-tetrahydroxytropane).* The ^{13}C-NMR spectral analysis of *N*-methylcalystegine B_2 revealed the presence of four methines (δ = 65.8, 74.9, 78.0 and 78.6 ppm), two methylenes (δ = 22.4 ppm and 29.3 ppm), a methyl (δ = 35.0 ppm), and a quaternary carbon (δ =95.9 ppm). A singlet signal (δ = 2.35 ppm, 3H) in the ^{1}H-NMR spectrum in ^2H_2O, together with the chemical shift of a methyl carbon signal in ^{13}C-NMR, was identified as an *N*-methyl group. These data suggested that this alkaloid was a tetrahydroxytropane with a *N*-methyl group from a natural source. This was also corroborated by the HRFAB MS analysis (*m/z* 190.1078 $[M + H]^+$, $C_8H_{16}O_4N$ requires 190.1079). In the ^{1}H-NMR spectrum, the large *J* values ($^3J_{2,3} = {}^3J_{3,4}$ = 8.5 Hz) in the H2, H3, and H4 signals indicate an all *trans*-axial orientation of them. In addition, NOE effects between N-CH_3 and H2 and between N-CH_3 and H4 show that the N-methyl group is axially oriented relative to the piperidine ring. The stereoconfiguration was corroborated by NOE effects between H3 and H6endo and between H3 and H7endo, and the presence of a W-path long-range coupling ($^4J_{2,7exo}$ = 1.5 Hz). Thus, the relative configuration of *N*-methylcalystegine B_2 was shown to be 1α,2β,3α,4β-tetrahydroxytropane. Goldmann et al. (1996) have chemically synthesized *N*-methylcalystegine B_2. However, since the physicochemical data

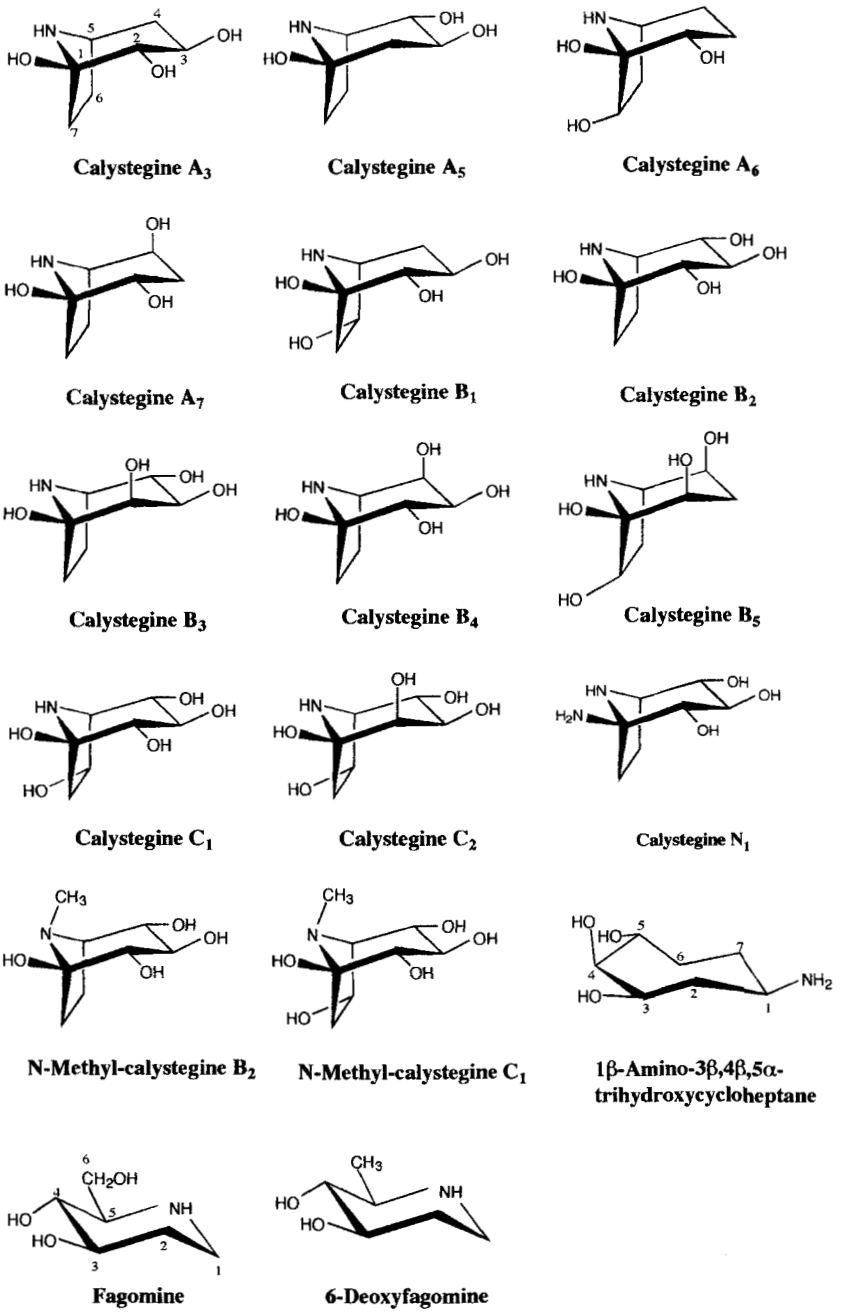

Fig. 1. Structures of polyhydroxylated alkaloids isolated from *L. chinense*.

have not been reported, we performed the full assignment of all carbons in *N*-methylcalystegine B_2 (see Experimental Procedures). In the ^{13}C-NMR spectrum, the N-methylation of calystegine B_2 produced 2.7-ppm and 7.2-ppm downfield shifts for C1 and C5 at the bridgehead, respectively, while 1.8−2.7-ppm upfield shifts for C2, C4, C6, and C7 were observed, and the chemical shift of C3 was little changed.

N-Methylcalystegine C_1 (1α,2β,3α,4β,6α-pentahydroxytropane). The results of ^{13}C-NMR and HRFAB MS (*m/z* 206.1028 $[M + H]^+$, $C_8H_{16}O_5N$ requires 206.1028) analyses indicate that this alkaloid is a pentahydroxytropane. In the ^{1}H-NMR in ^2H_2O, the presence of the N-methyl group (δ = 2.49 ppm, s, 3H) was observed. The coupling patterns of H2 (δ = 3.48 ppm, dd, $^3J_{2,3}$ = 8.8, $^4J_{2,7exo}$ = 1.8 Hz) and H3 (δ = 3.16 ppm, t, $^3J_{2,3}$ = $^3J_{3,4}$ = 8.8 Hz) indicate an all *trans*-axial orientation of H2, H3, and H4. Furthermore, an NOE effect between H3 and H6 suggested an endo orientation of H6, and NOE effects between N-CH_3 and H2 and between N-CH_3 and H4 show that the *N*-methyl group is axially oriented relative to the piperidine ring. Thus, the relative configuration of *N*-methylcalystegine C_1 was showed to be 1α,2β,3α,4β,6α-pentahydroxytropane.

1β-Amino-3β,4β,5α-trihydroxycycloheptane. Seven resonances (δ = 30.6, 33.9, 40.4, 51.2, 71.2, 75.7 and 80.8 ppm) in the ^{13}C-NMR spectrum of this alkaloid were very close to those (δ = 27.5, 34.1, 42.1, 57.5, 69.4, 72.3 and 83.8 ppm) of 1β-amino-2α,3β,5β-trihydroxycycloheptane, which we isolated previously from *P. alkekengi* var. *francheti* (Asano et al., 1996c). The HRFAB MS (*m/z* 162.1134 $[M + H]^+$) data established this alkaloid as an isomer of 1β-amino-2α,3β,5β-trihydroxycycloheptane. NOE effects between H1 and H3 or H6α and between H5 and H7β indicate that the seven-membered ring is in a chair conformation and H1, H3, and H5 are in α, α, and β orientations,

Table 1. Measured K_i values of selected calystegines for competitive inhibition of almond β-glucosidase and bovine liver β-galactosidase. Inhibition constants (K_i) were determined by Lineweaver-Burk plots. K_m values for *p*-nitrophenyl β-D-glucoside and galactoside were 3.6 mM and 0.82 mM, respectively, n.i., no inhibition.

Inhibitor	K_i for	
	β-glucosidase	β-galactosidase
	μM	
Calystegine A_3	20	30
Calystegine A_5	n.i.	n.i.
Calystegine B_1	1.8	1.6
Calystegine B_2	1.2	46
Calystegine B_3	200	n.i.
Calystegine B_4	7.3	n.i.
Calystegine C_1	0.45	3.6
Calystegine N_1	34	n.i.
N-Methylcalystegine B_2	n.i.	n.i.
N-Methylcalystegine C_1	n.i.	n.i.

respectively. In addition, a W-path long-range coupling between H2α and H4 shows that H4 is in an α orientation. Thus, the relative configuration of this alkaloid was determined to be 1β-amino-3β,4β,5α-trihydroxycycloheptane.

6-Deoxyfagomine. ^{13}C-NMR showed the presence of three methines, two methylenes, and a single methyl group, and decoupling experiments suggested that the methyl group was located at the 6 position. In the ^{1}H-NMR spectrum, the large *J* values ($^3J_{2,3} = {}^3J_{3,4} = 9.0$, $^3J_{4,5} = 9.6$ Hz) seen in the signals of H3 and H4 indicate an all *trans*-axial orientation of H3, H4, and H5. An NOE effect between H1ax and H3 or H5 indicates that the six-membered ring is in a chair conformation. Thus, the structure of this alkaloid was determined to be 6-deoxyfagomine.

Inhibition of β-glucosidase by calystegines. The kinetic constants of selected calystegines for competitive inhibition of almond β-glucosidase are shown in Table 1. An increase in degree of hydroxylation of the nortropane ring results in enhanced potency of inhibition against β-glucosidase, as seen in K_i values of calystegines A_3, B_1, B_2 and C_1. However, it is required for optimal inhibitory activity that the OH groups on the six-membered ring are all equatorial. An *exo* OH substituent at the 6 position enhances the potency. The replacement of an OH group at the bridgehead in calystegine B_2 by an NH_2 group lowered the potency about 30-fold. The N-methylation of calystegines B_2 and C_1 removed their inhibition of this enzyme. The lack of inhibition of calystegine A_5 toward all glycosidases tested suggests that the presence of the C2 OH group is an essential feature for recognition and strong binding by the active site of glycosidases.

Inhibition of β-galactosidase by calystegines. The kinetic constants of selected calystegines for competitive inhibition of bovine liver β-galactosidase are shown in Table 1. The lack of inhibition of calystegines B_3 and B_4 suggests that the equatorial orientation of all OH groups on the six-membered ring is an essential feature for β-galactosidase inhibition. From a comparison of the potency of calystegines A_3, B_1, B_2 and C_1, the presence of the C4 OH group does not appear to be essential for binding to this enzyme and an additional hydroxylation at C6exo enhances the potency 10-fold. The replacement of an OH group at the bridgehead by an NH_2 group, and the N-methylation completely remove any inhibitory activity.

Table 2. Measured K_i values and modes of inhibition of selected calystegines against pig kidney trehalase. K_m was 5.5 mM for trehalose. n.i., no inhibition.

Inhibitor	K_i	Inhibition mode
	μM	
Calystegine A_3	5.3	competitive
Calystegine A_5	n.i.	n.i.
Calystegine A_7	22	competitive
Calystegine B_1	n.i.	n.i.
Calystegine B_2	5.3	competitive
Calystegine B_3	25	competitive
Calystegine B_4	1.2	competitive
Calystegine C_1	16	competitive
Calystegine N_1	62	noncompetitive
N-Methylcalystegine B_2	90	competitive
N-Methylcalystegine C_1	n.i.	n.i.

Table 3. Measured K_i values of selected calystegines for competitive inhibition of coffee bean α-galactosidase. K_m was 1.3 mM for *p*-nitrophenyl-α-D-galactosidase. n.i., no inhibition.

Inhibitor	K_i
	μM
Calystegine A_3	20
Calystegine A_5	n.i.
Calystegine B_1	n.i.
Calystegine B_2	0.86
Calystegine B_3	n.i.
Calystegine B_4	n.i.
Calystegine C_1	90
Calystegine N_1	75
N-Methylcalystegine A_3	5.2
N-Methylcalystegine B_2	0.47
N-Methylcalystegine B_3	62
N-Methylcalystegine B_4	36
N-Methylcalystegine C_1	93

Inhibition of trehalase by calystegines. Measured K_i values and modes of inhibition toward pig kidney trehalase are shown in Table 2. From a comparison of the potency of calystegines A_3, B_2 and B_4, the C4 equatorial OH group does not appear to be essential for binding to this enzyme, whereas its axial inversion appears to enhance an affinity (Asano et al., 1996b). The K_i values of calystegines A_7 and B_4 indicate that the deoxygenation at C3 lowers potency about 20-fold. The additional hydroxylation at C6exo and the N-methylation result in a significant decrease of affinity. Replacement of the bridgehead OH group at C1 by an NH_2 group, as in calystegine N_1, reduces the inhibitory activity by one order of magnitude in comparison with calystegine B_2. Moreover the inhibition by calystegine B_2 is of the competitive type, whereas calystegine N_1 is a non-competitive inhibitor (Asano et al., 1996a).

Inhibition of α-galactosidase by calystegines. The kinetic constants of natural calystegines and synthetic N-methyl derivatives for competitive inhibition of coffee bean α-galactosidase are shown in Table 3. Calystegines B_1 and C_1 are potent inhibitors of β-galactosidase, with K_i values of 1.6 μM and 3.6 μM, respectively, but have no inhibitory activity or a weak effect on α-galactosidase. On the other hand, calystegines A_3 and B_2, which have the same substitution pattern and stereochemistry other than the absence of the C4 OH group, are inhibitors of α-galac-

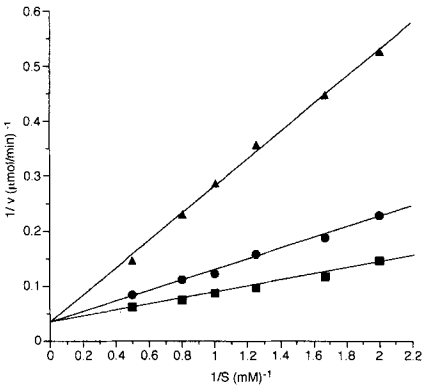

Fig. 2. Lineweaver-Burk plots of *N*-methylcalystegine B_2 inhibition of coffee bean α-galactosidase. Increasing concentrations of *p*-nitrophenyl-α-D-galactoside were used to determine the K_m and K_i values. Concentrations of *N*-methylcalystegine B_2 were 0 (■), 0.5 μM (●) and 2 μM (▲). The data were plotted as $1/V$ against 1/[S]. The calculated K_m was 1.3 mM.

Table 4. Concentration of selected calystegines giving 50% inhibition of rat liver lysosomal glycosidases. n.i., less than 50% inhibition at 1000 μM.

Inhibitor	IC_{50} for			
	β-glucosidase	α-galactosidase	β-galactosidase	β-xylosidase
	μM			
Calystegine A_3	90	n.i.	n.i.	850
Calystegine B_1	4.6	n.i.	n.i.	120
Calystegine B_2	15	21	n.i.	n.i.
Calystegine C_1	3.6	n.i.	n.i.	n.i.
N-Methylcalystegine B_2	n.i.	7.4	n.i.	n.i.
N-Methylcalystegine C_1	n.i.	n.i.	n.i.	n.i.

tosidase, with K_i values of 20 μM and 0.86 μM, respectively. These results suggest that the C6exo OH group in calystegines B_1 and C_1 interferes with their binding to α-galactosidase. Replacement of the bridgehead OH group at C1 by an NH_2 group reduces the inhibitory activity toward this enzyme by about two orders of magnitude in comparison with calystegine B_2. The N-methylation of calystegine B_2 abolished its inhibition of β-galactosidase, whereas activity toward α-galactosidase was somewhat enhanced. The inhibition was of the competitive type (Fig. 2). More recently, Goldmann et al. (1996) reported that N-methylation of natural calystegine B_2 suppresses inhibition of β-glucosidase but retains a potency toward α-galactosidase. Calystegines B_3 and B_4 are not inhibitors of α-galactosidase, while synthetic *N*-methylcalystegines B_3 and B_4 inhibited this enzyme in a competitive manner, with K_i values of 62 μM and 36 μM, respectively. The N-methylation of calystegines enhanced the potency toward α-galactosidase but failed to have any activity toward other glycosidases. Thus, methylation of the nitrogen to give tropane analogs altered the specificity and potency of the inhibitors.

Inhibition of lysosomal glycosidases by calystegines. The occurrence of calystegines in the leaves, skins, and sprouts of potatoes (*S. tuberosum*), in fruits of the egg plants (*S. melongena*), and in roots of sweet potato (*I. batatas*), raises concerns regarding the safety of these vegetables in the human and animal diet (Nash et al., 1993; Dräger, 1995; Molyneux et al., 1996) with regard to the inhibition of lysosomal enzymes (Stegelmeier et al., 1995). We therefore examined the inhibitory activities of calystegines toward rat liver lysosomal glycosidases. The IC_{50} values of selected calystegines toward lysosomal glycosidases are shown in Table 4. Calystegines B_1 and C_1 were potent inhibitors of rat liver lysosomal β-glucosidase, with IC_{50} values of 4.6 μM and 3.6 μM, respectively. Calystegine B_2 was a good inhibitor of this enzyme, but the N-methyl derivatives of calystegines B_2 and C_1 lost potency toward this enzyme. *N*-Methylcalystegine B_2 showed stronger inhibition than did the parent compound toward rat liver lysosomal α-galactosidase.

DISCUSSION

One of the aspects of the biological activity of glycosidase inhibitors in plants is their potential toxicity toward animals or humans. Cattle eating *Astragalus*, *Oxytropis* or *Swainsona* species are known to develop the signs of poisoning, analogous to those of genetic mannosidosis, resulting in accumulation of mannose-rich oligosaccharides in lysosomes and neuronal vacuolation (Dorling et al., 1978). These poisonings have been shown to be due to swainsonine, which is a potent inhibitor of α-mannosidase. Castanospermine, which occurs in *Castanospermum australe*, is a potent inhibitor of α-glucosidase, and the signs of poisoning of livestock and humans are similar to those observed for Pompe's disease, arising from a genetic deficiency of lysosomal α-glucosidase. Feeding experiments of castanospermine with rats result in glycogen accumulation (Saul et al., 1985) and degenerative vacuolation of hepatocytes and skeletal myocytes (Stegelmeier et al., 1995). Calystegines are polyhydroxylated bicyclic amines with structural affinities to the indolizidine alkaloids, swainsonine and castanospermine. Calystegines are tropane alkaloids which were characterized by the absence of an *N*-methyl group, but in this work *N*-methylcalystegines B_2 and C_1 were isolated as very minor components

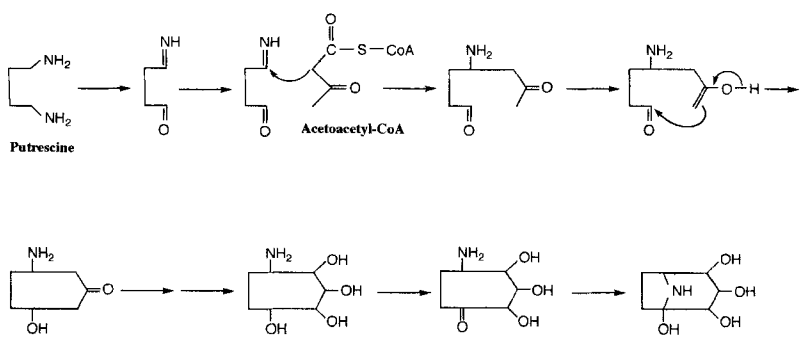

Fig. 3. Postulated biosynthesis of the tetrahydroxynortropane.

from *L. chinense* with 12 other calystegines. Calystegines A_3, B_1, B_2 and C_1 inhibit rat liver lysosomal β-glucosidase, with IC_{50} values ranging from 10^{-4} M to 10^{-6} M (Table 4). In particular, calystegines B_1 and C_1 are potent competitive inhibitors of this enzyme, with K_i values of 1.9 μM and 1.0 μM, respectively. It would not be unreasonable to predict that these calystegines would produce syndromes that mimic Gaucher's disease, which is a condition characterized by the widespread accumulation in histocytes of glucocerebroside, a glycolipid made up of equimolar amounts of ceramide and D-glucose. Calystegines A_3 and B_2 and *N*-methylcalystegines are good inhibitors of coffee bean α-galactosidase (Table 3). Among these alkaloids, *N*-methylcalystegine B_2 is a very specific inhibitor of α-galactosidase, lacking other glycosidase inhibitory activities, and inhibits rat liver lysosomal α-galactosidase with a K_i value of 1.8 μM. An absence of α-galactosidase in humans is responsible for the lysosomal storage disorder, Fabry's disease. In this disease there is widespread storage of neural glycolipids consisting predominantly of ceramide trihexose [ceramide-D-glucose-(D-galactose)$_2$] in the blood vessels, ganglion cells, skin, heart, kidneys and elsewhere. As a specific and potent inhibitor of this enzyme, *N*-methylcalystegine B_2 might serve to chemically induce a similar deficiency, providing a useful experimental model for the genetic disease.

Calystegines are nortropane alkaloids with a high degree of hydroxylation and an unusual aminoketal functionality at the bridgehead position. The biosynthesis of the calystegines has not been elucidated but a reasonable working hypothesis would involve modification of the tropane alkaloid biosynthetic pathway, which commences with tropinone derived from L-ornithine and/or L-arginine (Robins et al., 1994). Dräger (1995) has proposed that pseudotropine, formed from tropinone through reduction by tropinone reductase II, could be the intermediate of all of the calystegines, since a common feature is the presence of the equatorial OH group at C3. However, a number of difficulties exist with this proposal. A profound problem is the presence of an unusual aminoketal functionality at the bridgehead position and the high degree of hydroxylation. The isolation of 1β-amino-2α,3β,5β-trihydroxycycloheptane from *P. alkekengi* var. *francheti* and 1β-amino-3β,4β,5α-trihydroxycycloheptane from *L. chinense* may indicate that calystegines are derived by a divergent pathway of the tropane alkaloid biosynthesis that does not involve the formation of tropinone or pseudotropine (Fig. 3), or by a similar pathway. Alternatively, the calystegines could result from enzymic oxidation of the 5-OH group of the polyhydroxylated 1-aminocycloheptanes. Calystegines A_6, A_7 and B_5 lack an hydroxyl group at C3. These calystegines might be generated from β-elimination by the carbonyl group resulting from this oxidation. The polyhydroxylated tropane alkaloids bearing an *N*-methyl group on a nitrogen atom have not been found in any plants other than *L. chinense*. Since in the tropane alkaloids N-methylation occurs at a very early stage of the biosynthetic pathway, *N*-methylcalystegines B_2 and C_1 might be derived by the same postulated pathway via *N*-methylputrescine. The biosynthetic pathway may be elucidated by experiments with transformed root cultures, since the advantages of using transformed roots are their independence of plant-growth regulators, their often rapid growth rates, and the ease of isolation of the calystegines from cultures.

Two polyhydroxylated piperidine alkaloids, fagomine and 6-deoxyfagomine, were also isolated from *L. chinense*. Previously, we reported the occurrence of fagomine in the leaves and roots of *Morus* spp. (Moraceae) (Asano et al., 1994a,b) and in the leaves of *Xanthocercis zambesiaca* (Leguminosae) (Kato et al., 1997b). Fagomine was found to have a potent antihyperglycemic effect in streptozocin-induced diabetic mice and to potentiate markedly immunoreactive insulin release (Kimura et al., 1995).

Swainsonine, an inhibitor of α-mannosidase II during N-linked glycoprotein processing, is known to show potential as a therapeutic agent in the treatment of cancer. Interleukin(IL)-2 is a secreted glycoprotein that acts as an activation and proliferative signal for lymphocytes expressing membrane-bound-glycoprotein IL-2 receptors. Swainsonine enhances lymphocyte IL-2-receptor expression and IL-2-induced proliferation after mitogen stimulation (Bowlin and Sunkara, 1988), and induces tumoricidal activity and secretion of IL-1 in macrophages (Grzegorzewski et al., 1989). Castanospermine, an inhibitor of processing α-glucosidase I, has been shown to exhibit antimetastatic activity by inhibiting platelet aggregation of metastatic cells as well as reducing adhesion of tumor cells to the vascular endothelium (Spearman et al., 1991; Humphries et al., 1986), in addition to antiretroviral potency by inhibiting replication of human immunodeficiency virus (Walker et al., 1987). *N*-Butyldeoxynojirimycin, which is a potent inhibitor of processing α-glucosidase I and an anti-(human immunodeficiency virus) agent, is an inhibitor of the glucosyltransferase-catalyzed biosynthesis of glucosylceramide and prevents lysosomal glycolipid storage (Platt et al., 1994). Therefore, this compound offers a therapeutic approach for the management of this and other glycolipid-storage disorders. The possibilities for the existence of equivalent properties among the calystegines and their derivatives should be vigorously explored.

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